ARTIFICIAL ANTIBODIES, METHOD OF PRODUCING THE SAME AND USE THEREOF

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The present invention concerns artificial antibodies, a method for producing the artificial antibodies, a method for determination of an organic molecule in a fluid sample, a method for separation or isolation of an organic molecule and use of the latter methods in immunoassays as well as a method of therapy or diagnostics.

Antibodies are used in several areas, such as therapy, immunoaffinity, purification and in particular in immunoassays. As to the latter aspect the corresponding antigens can either be small or large molecules.

Antibodies are normally produced by immunising animals with the corresponding antigen leading to polyclonal antibodies, or by using fused cells (B cells) allowing the obtained cell lines to produce monoclonal antibodies.

Recent efforts in obtaining other biologically derived antibodies or at least antibody-like compounds involve recombinant techniques applied to bacteria or plants.

Antibodies can be raised against most compounds; they are versatile reagents employed in numerous applications[1-5], ranging from basic research to clinical analysis. However, being bio-macromolecules they require careful handling and their production is costly[5].

A potentially useful alternative would be the production of non-biologically derived antibody mimics or artificial antibodies, such as polymer structures that are similar to biological antibodies in binding and recognising antigens.

The inherent advantages of such systems would be that the need for animal sources is obliviated, and that antibody mimics can be obtained for cases where it is difficult or impossible to raise antibodies, as for immuno suppressive agents, such as cyclosporin, certain structures, such as macrolides or short peptides.

Furthermore, such non-biological systems could be made more stable, allowing repeated use, higher temperatures and easy sterilisation.

In addition the need for derivatisation of antigens for immunisation purposes is made unnecessary, thereby avoiding the often complicated chemistry and sometimes decreased recognition for the original target molecule (= antigen).

Since the development of the first radioimmunoassay[1], immunological techniques using labelled reactants have gained an extraordinary prominence in the field of medical research and in clinical diagnosis. In particular, the discovery of monoclonal antibodies[2] and their use in immunoassays has offered novel advantages and more possibilities. Despite the plethora of markers and different procedures[3,4] that have been employed, all the immunological techniques exploit the remarkable affinity and specificity of antibodies. However, antibodies are labile biomolecules which require careful handling and storage. Their production is a time-consuming procedure[5], including several laborious steps like conjugation of the hapten to a carrier protein, immunisation of animals and isolation of immunoglobulins.

Thus, there was a need for an immunoassay-like technique in which stable and easily prepared highly selective polymers, rather than antibodies are used.

The technique of molecular imprinting has attracted much attention in the last few years[6-8]. Recently, molecular imprinting has been developed to a stage of practical application in enantiomeric separations[11-15], in particular in the resolution of racemic drugs such as β-blockers[16].

Furthermore, the technique has been applied to make synthetic enzymes[9,10].

The technique of molecular imprinting and its special form of non-covalent imprinting as developed by the inventors makes it possible to achieve the above objects.

Briefly, the technique involves polymerisation of functional monomers in the presence of a print molecule (see Scheme 1). Subsequent removal of the print molecule from the rigid polymer results in sites within the polymer that are complementary to and have an affinity for the original print molecule.

According to the invention there are provided artificial antibodies, which consist of polymers that carry specific binding sites mimicking the properties of antibodies.

There is also provided, according to another aspect of the invention, a method for producing artificial antibodies, in which polymerisable monomers carrying functional groups and crosslinking monomers are polymerised in the presence of a print molecule and subsequently the print molecule is removed leaving specific binding sites complementary to the print molecule.

The invention also provides for a method for determination of an organic molecule in a fluid sample. According to this method, a known amount of the organic molecule provided with a label is added to the sample, the sample is contacted with artificial antibodies having specific binding sites for the organic molecule, whereby the labelled and unlabelled organic molecules are competitively bound to the binding sites, and the labelled organic molecule is determined either unbound in the supernatant or bound by the polymer.

There is also provided a method for separation or isolation of an organic molecule from a fluid sample, in which the sample, labelled or not, is contacted with an excess of artificial antibodies consisting of a polymer having specific sites for the organic molecule, whereby the organic molecule is bound to the binding sites, and optionally the organic molecule is measured bound to the artificial antibodies or eluted from the antibodies.

The invention also provides for a method of therapy or diagnosis, in which artificial antibodies are administrated to a mammal body, which artificial antibodies consist of a biocompatible polymer carrying specific binding sites mimicking the properties of antibodies towards an organic molecule.

In one embodiment of the invention, the polymers are prepared by non-covalent polymerisation.

The polymers constituting the artificial antibodies are preferably built up of polymerisable monomers carrying functional groups and crosslinking monomers. Preferably the polymerisable monomers carrying functional groups are chosen among negatively charged monomers such as methacrylic acid, itaconic acid, basic monomers such as vinylpyridine, vinylimidazole, hydrophobic monomers carrying alkyl chains, monomers allowing π-π-interactions, van der Waals forces.

In one embodiment of the invention, polymers are built up of methacrylic acid crosslinked by ethylene glycol dimethacrylate.

If the artificial antibodies are to be used for administration to a mammal body the polymers must be biocompatible. Preferably they must be of the size not more than 5 µm or the size of normal biological antibodies, most preferred 10-100 nm.

In preparation of artificial antibodies according to the invention, the polymer is ground to a particle size of normally ~ 25 µm for use in so-called heterogenous assays.

The fines, that is particles with a size of 10-100 or 1000 nm, resulting from the grinding, can be kept in solution or suspension and used for instance in so-called homogenous immunoassays. Such assays are extremely sensitive and can be performed involving e.g. two different antibodies.

Another advantage with the fine particles is that they are more suitable for use in therapy or diagnostics.

Preferably the binding sites are specific for a compound chosen from the group consisting of drugs, metabolites, nucleotides, nucleic acids, carbohydrates, proteins, hormones, toxins, steroids, prostaglandins and leukotrienes.

In one embodiment the binding sites are specific for theofylline or diazepam.

Suitable labels for use in the methods according to the invention are radioligands, enzymes, biotin, steroids, fluorochromes, gold.

The methods according to the invention are preferably used in immunoassays, especially in radioimmunoassays.

The method of therapy or diagnosis according to the invention comprises several different modes of action. For example, it can be used to withdraw an undesired organic molecule from a mammal body, such as a toxin. In another embodiment the artificial antibodies assemble around a cancer cell to indicate the presence of such a cell. In a further embodiment the artificial antibodies are bringing a drug to specific targets, for instance cancer cells.

In one embodiment of treating a mammal body an extra corporal device containing the artificial antibodies is coupled to the body via a shunt in the bloodstream, and the bloodstream is passed through the device.

For the studies the inventors chose two chemically unrelated drugs, theophylline and diazepam, as print molecules. Theophylline, a commonly used drug in the prevention and treatment of asthma, apnea and obstructive lung diseases, has a narrow therapeutic index (56-112 µmol L^{-1} serum) requiring careful monitoring of serum concentrations[17]. Diazepam (e.g. valium) is a member of the benzodiazepine group of drugs widely used as hypnotics, tranquilizers and muscle relaxants[18]. Benzodiazepines are one of the most commonly implicated substances in drug overdose situations and their detection in body fluids is very useful in clinical and forensic toxicology. Current methods for measuring theophylline and benzodiazepines are

based on high-performance liquid chromatography (HPLC)[19-21] and on immunological techniques[22-26].

The polymers were prepared using methacrylic acid (MAA) as the functional monomer and ethylene glycol dimethacrylate (EDMA) as the crosslinking monomer (Scheme 1). This is a well characterised polymer system that has been used for the preparation of molecular imprints against a number of compounds [12-14,16]. The carboxylic acid function of MAA has been shown to form ionic interactions with amino groups[12] and hydrogen bonds with polar functionalities of the print molecule[14]. The inventors assume that hydrogen bonding is the predominant type of force operating during imprinting and subsequent recognition in the present system. Dipole-dipole and hydrophobic interactions may also contribute.

The solvent compositions giving optimal binding and selectivity were determined for each polymer (see Example 2 and Fig. 1 below). As a general guide[14,27]: i) in a more apolar solvent the substrate binds more strongly to the polymer than in polar solvents, and ii) small amounts of acetic acid can be added to the solvent in order to supress non-specific binding. The eqilibrium dissociation constants (K_D) for binding of the drugs to the corresponding polymers were estimated by Scatchard plot analysis using radio-labelled ligands. In both cases, the Scatchard plots were nonlinear and fitted well with two K_D values, for high and low affinity binding sites. The inventors believe that, as in the case of polyclonal antibodies, the polymers contain a heterogenous population of sites with different affinities for the print molecule. The K_D values for the high and low affinity binding sites, calculated with the LIGAND programme (Elsevier-Biosoft), were 3.46×10^{-7} M and 6.55×10^{-5} M (associated with a population of sites of 0.016 µmol g^{-1} and 1.28 µmol g^{-1}, respectively) for theophylline and 3.76×10^{-8} M and 7.36×10^{-8} M (0.0071 µmol g^{-1} and 0.51 µmol g^{-1}) for diazepam.

Polymers prepared against theophylline or diazepam were used as antibody-substitutes in the construction of competitive binding for theophylline and diazepam determination in human serum. The method, which we name Molecularly Imprinted Sorbent Assay (MIA), relies on the inhibition of binding of radio-labelled ligand by the serum analyte. The amount of radioligand bound to the polymer is inversely related to the concentration of drugs present in the sample. Drug free serum samples spiked with known amounts of theophylline or diazepam were used for establishing the standard calibration curves. Prior to the actual assay, the drug was extracted from the serum by standard protocols used for HPLC-analysis[19-21] (Fig. 1). The MIA for theophylline was linear over the range 14-224 μmol L^{-1} which is satisfactory for therapeutic monitoring of the drug. The results for diazepam were linear over the range which is normally used in standard immunoassay techniques for benzodiazepines (0.44-28 μmol L^{-1}).

The specificity of the method was tested by the determination of cross-reactivity of major metabolites and of drugs structurally related to theophylline or diazepam (Table 1).

TABLE 1 Cross-reactivity of various xanthine and uric acid derivatives for binding of ^{3}H-theophylline (bronchodilator) and various benzodiazepines for binding of ^{3}H-diazepam (tranqilizer) to artificial antibodies (ArtAb's) and natural antibodies (Ab's).

Theophylline antibodies			Diazepam antibodies		
Competitive ligand	Cross-reaction (%)		Competitive ligand	Cross-reaction (%)	
	ArtAb	Ab*		ArtAb	Ab**
Theophylline (1,3-dimethyl-xanthine)	100	100	Diazepam (e.g. valium)	100	100
3-Methylxantin	7	2	Alprazolam	40	44
Xanthine	<1	<1	Demethyldiazepam	27	32
Hypoxanthine	<1	<1			
7-(β-Hydroxyethyl)-1,3-di-methylxanthine	<1	<1	Clonazepam	9	5
Caffeine (1,3,7-trimetylxan-thine)	<1	<1	Lorazepam	4	1
Theobromine (3,7-dimetylxan-thine)	<1	<1	Chlordiazepoxid	2	<1
Uric acid	<1	<1			
1-Methyluric acid	<1	<1			
1,3-Dimethyluric acid	<1	<1			

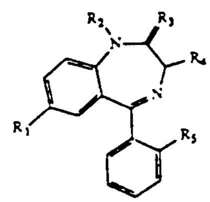
The ligands were added to drug free serum and assayed as described in Fig. 1. Cross--reactivities are expressed as the molar ratio of theophylline and diazepam, respectively, to ligand giving 50% inhibition of radioligand binding to polymer.

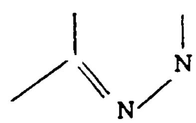
* Data from ref 22.

** Data from ref 24.

The MIA method for theophylline (1,3-dimethylxanthine) appears to be highly specific since from all the compounds tested only 3-methylxanthine showed some cross-reactivity.

In the case of the diazepam assay several other benzodiazepines showed significant cross-reactivity. This was, however, expected because benzodiazepines are very similar in structure, as seen below:



	R_1	R_2	R_3	R_4	R_5
Diazepam	Cl	Me	O	H	H
Desmethyldiazepam	Cl	H	O	H	H
Clonazepam	NO_2	H	O	H	Cl
Lorazepam	Cl	H	O	OH	Cl
Alprazolam	Cl			H	H

and even antibodies have difficulty in distinguishing between them[25,26] (Table 1).

The ability of the MIA method for accurate measurement of theophylline was evaluated by analysing 32 patient serum samples. The sample were also analysed with the Enzyme-Multiplied Immunoassay Technique (EMIT)[28] and the comparison of the results obtained showed excellent correlation between the two methods (Fig. 1). Furthermore, the reliability of the assay was determined by measurement of theophylline samples of known concentration (three clinical significant concentrations; eleven repetitions; coefficient of variation ≤ 6.5%).

The results presented here demonstrate, for the first time, the ability to use chemically prepared macromolecules with preselected specificity, instead of the traditional biomolecules, as receptors in competitive binding assays. A great advantage of molecularly imprinted polymers is their simple and rapid (two to three days) preparation and their remarkable stability. They can be stored in the dry state, even at elevated temperatures, for several years without loss of recognition capabilities[27]. In addition, the potential to reuse the polymers may prove valuable. Furthermore, by analogy to immunoaffinity chromatography, molecularly imprinted polymers could be useful for the separation and isolation of different compounds. Apart from the practical importance of the described preparations, structural studies on the interactions of drugs with their artificial receptors could yield valuable insight into the nature of molecular recognition phenomena[29-31].

Molecular imprints may be obtained against functionality complementary to the monomer[14,27]. There is a potential for molecularly imprinted artificial antibodies in the analysis of many other drugs, metabolites, hormones, toxins, etc.

It is also noteworthy that molecularly imprinted polymers provide a potential alternative to the use of laboratory animals for the production of antibodies. Preliminary data from similar studies with an emphasis on recognition in aqueous systems using other compounds such as opiates and biologically active peptides, indicate that this technique promises to become widely useful.

The invention is described more in detail with reference to the following examples and the accompanying drawing.

Figure 1 shows a comparision of the competitive binding assays Enzyme-Multiplied Immunoassay Technique (EMIT)[28] and MIA for determination of serum concentration av theophylline in patient samples (n=32).

Example 1

Preparation of molecularly imprinted polymers

The preparation follows the reaction of Scheme 1.

A) The functional monomer, methacrylic acid (MAA,1), is mixed with the print molecule, here theophylline (2), and ethylene glycol dimethacrylate (EDMA), the crosslinking monomer, in a suitable solvent. MAA is selected for its ability to form hydrogen bonds with a variety of chemical functionalities of the print molecule.

B) The polymerisation reaction is started with the addition of initiator (AIBN) and a rigid insoluble polymer is formed. "Imprints", which are complementary in both shape and chemical functionality to the print molecule, are now present within the polymeric network.

C) The print molecule is removed by extraction.

The wavy lines in Scheme 1 represent an idealised polymer structure but do not take into account the accessibility of the substrate to the recognition site in the macroporous polymer structure.

METHODS

Anti-theophylline polymer

To a glass bottle were added chloroform (250 ml), theophylline (4.7 g), MAA (9 g), EDMA (93,5 g) and 2,2'--azobis(2-methylpropionitrile) (AIBN, initiator, 1.2 g). The mixture was degassed under vacuum in a sonicating waterbath and sparged with nitrogen for 5 min. The polymerisation reaction took place at 60°C for 24 h. The bulk polymer was grounded in a mechanical mortar and wet sieved (water) through a 25 µm sieve. The fines were removed by repeated settling in acetonitrile. The print molecule (theophylline) was extracted by extensive washing of the particles with methanol-acetic acid (9/1, v/v). Finally, the polymer particles were dried under vacuum and stored in a desiccator.

Anti-diazepam polymer

Diazepam (1.27 g) was mixed with MAA (2.26 g), EDMA (26.1 g) and AIBN (0.5 g) in chloroform (39 ml). The polymerisation mixture was degassed under vacuum in a sonicating water-bath, sparged with nitrogen and then polymerised under UV (366 nm) at 4°C for 16 h. The resulting polymer was then treated as described above.

Example 2

A comparison of the competitive binding assays Enzyme-Multiplied Immunoassay Technique (EMIT)[28] and MIA for determination of serum concentration of theophylline in patient samples (n=32) was performed. EMIT reagents were supplied by the manufacturer (SYVA, Palo Alto, USA). All enzyme immunoassays were preformed at the department of Clinical Pharmacology, University Hospital, Lund, Sweden, according to the method of the manufacturer. The result is shown in Fig. 1:
Slope: 0.99, Intercept: 1.50 μmol L^{-1}, correlation coefficient: 0.98.

METHODS

The assay conditions were established by applying similar protocols as is standard for the optimisation of immunoassays using antibodies[32]. 40 μl of each sample was mixed with 40 μl of HCl (0.2 M) and extracted with 1 ml of dichloromethaneisopropanol (4/1, v/v). The organic layer was evaporated at 40°C under a stream of nitrogen. The residue was redissolved in 100 μl of acetonitrile-acetic acid (99/1, v/v) containing [^{3}H]-theophylline (5 ng, 18.6 Ci $mmol^{-1}$). Polymer imprinted against theophylline was then added (12.5 mg of polymer in 0.9 ml of the same solvent) and the mixture was incubated for 15 h at room temperature. The binding equilibrium was reached after 8 h, 80 and 90% of the binding occurred within 3 and 5 h. After centrifugation, the unbound [^{3}H]-theophylline in 200 μl of the supernatant was measured by liquid scintillation counting. The calibration graph was linear over the range 14-224 μmol L^{-1} (correlation coefficient = 0.999)

and the detection limit of the assay was found to be 3.5 μmol L^{-1}. The diazepam assay, performed in a similar manner using 5 mg of polymer in toluene-heptane (4:1; v/v), was linear from 0.44 to 28 μmol L^{-1} (correlation coefficient = 0,991) with a detection limit of 0.2 μmol L^{-1}.

References

1. Yalow, R. S. & Berson, S. A. Nature 184, 1648-1649 (1959).
2. Köhler, G. & Milstein, C. Nature 256, 495-497 (1975).
3. Oellerich, M. J. Clin. Chem. Clin. Biochem. 22, 895-904 (1984).
4. Gosling, J. P. Clin. Chem. 36, 1408-1427 (1990).
5. Kurstak, E. in Enzyme Immunodiagnosis (ed Kurstak, E.) 5-11 (Academic Press, London, 1986).
6. Ekberg, B. & Mosbach, K. Trends Biotechnol. 7, 92-96 (1989).
7. Wulff, G. Amer. Chem. Soc. Symp. Series 308, 186-230 (1986).
8. Shea, K. J. & Sasaki, D. Y. J. Am. Chem. Soc. 113, 4109-4120 (1991).
9. Robinson, D. K. and Mosbach, K. J. Chem. Soc. Chem. Commun. 14, 969-970 (1989).
10. US patent No. 5,110,833 to Klaus Mosbach.
11. Sellergren, B., Ekberg, B. & Mosbach, K. J. Chromatogr. 347, 1-10 (1985).
12. Sellergren, B., Lepistö, M. & Mosbach, K. J. Am. Chem. Soc. 110, 5853-5860 (1988).
13. O'Shannessy, D. J., Ekberg, B., Andersson, L. I. & Mosbach, K. J. Chromatogr. 470, 391-399 (1989).
14. Andersson, L. I. & Mosbach, K. J. Chromatogr. 516, 313-322 (1990).
15. Wulff, G. & Minarik, M. J. Liq. Chromatogr. 13, 2987-3000 (1990).
16. Fischer, L., Müller, R., Ekberg, B. & Mosbach, K. J. Am. Chem. Soc. 113, 9358-9360 (1991).
17. Hendeles, L., Weinberger, M. & Johnson, G. Clin. Pharmacokinetics 3, 294-312 (1978).
18. Harvey, S. L. in The Pharmacological Basis of Therapeutics (eds Gilman, A. G., Goodman, L. S., Rail, T. W. & Murad, F.) 339-351 (Marcel Dekker Inc., New York, 1985).

19. Meffin, P. J. & Miners, J. O. in Progress in Drug Metabolism (eds Bridges, J. W. & Chasseaud, L. F.) Vol. 4, 261-307 (J. Wiley, London, 1980).

20. Peng, G. W., Gadalla, M. A. F. & Chiou, W. L. Clin. Chem. 24, 357-361 (1978).

21. Mura, P., Piriou, A., Fraillon, P., Papet, Y. & Reiss, D. J. Chromatogr. 416, 303-310 (1987).

22. Castro, A., Ibanez, J., Voight, W., Noto, T. & Malkus, H. Clin. Chem. 24, 944-946 (1978).

23. Chang, J., Gotcher, S. & Gushaw, J. B. Clin. Chem. 26, 361-367 (1982).

24. Ponceiet, S. M., Limet, J. N., Noel, J. P., Kayaert, M. C., Galanti, L. & Collet-Cassart, D. J. Immunoassay, 11, 77-88 (1990).

25. Baselt, R. C. in Advances in Analytical Toxicology (ed Baselt, R. C.) Vol. 1, 81-123 (Biomedical Publications, Foster City, CA, 1984).

26. Aitunkaya, D. & Smith, R. N. Forensic. Sci. Int., 39, 23-37 (1988).

27. Andersson, L. I. thesis, Lund Univ. (1991).

28. Dietzler, D. N., Waldner, N., Tieber, V. L., McDonald, J. M., Smith, C. H., Ladenson, J. H. & Leckie, M. P. Clin. Chim. Acta 101, 163-181 (1980).

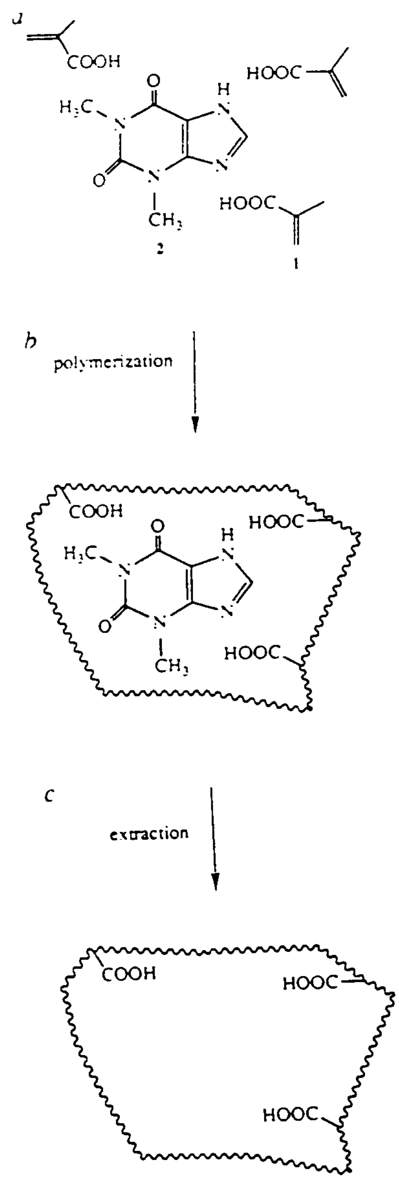
29. Cram, D. J. Nature 356, 29-36 (1992).

30. Rebek, J. Jr. Angew. Chem. Int. Ed. Engl. 29, 245-255 (1990).

31. Desiongchamps, G., Galán, A., de Mendoza, J. & Rebek, J. Jr. Angew. Chem. Int. Ed. Engl. 31, 61-63 (1992).

32. Tijssen, P. Laboratory Techniques in Biochemistry and Molecular Biology, Pratice and Theory of Enzyme Immunoassays 5th printing (Elsevier Publishers B.V., Amsterdam, 1988).

SCHEME 1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COOH
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H
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CH_3
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b
polymerization
COOH
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c
extraction
COOH
HOOC
HOOC